

# Do Pharmacological Approaches that Prevent Opioid Tolerance Target Different Elements in the Same Regulatory Machinery?

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**Abstract:** In the nervous system, the interaction of opioids like heroin and morphine with the G protein-coupled Mu-opioid receptor (MOR) provokes the development of tolerance to these opioids, as well as physical dependence. Tolerance implies that higher doses of these drugs must be consumed in order to obtain an equivalent sensation, a situation that contributes notably to the social problems surrounding recreational opioid abuse. The mechanisms that promote opioid tolerance involve a series of adaptive changes in the MOR and in the post-receptor signalling elements. Pharmacological studies have consistently identified a number of signalling proteins relevant to morphine-induced tolerance, including the delta-opioid receptor (DOR), protein kinase C (PKC), protein kinase A (PKA), calcium/calmodulin-dependent kinase II (CaMKII), nitric oxide synthase (NOS), N-methyl-D-aspartate acid glutamate receptors (NMDAR), and regulators of G-signalling (RGS) proteins. Thus, it is feasible that these treatments which diminish morphine tolerance target distinct elements within the same regulatory machinery. In this scheme, the signals originated at the agonist-activated MORs would be recognised by elements such as the NMDARs, which in turn exert a negative feedback on MOR-evoked signalling. This process involves DOR regulation of MORs, MOR-induced activation of NMDARs (probably *via* the regulation of Src, recruiting PKC and  $G\alpha$  subunits) and the NMDAR-mediated activation of CaMKII. The active CaMKII promotes the sequestering of morphine-activated  $G\beta\gamma$  dimers by phosducin-like proteins (PhLP) and of  $G\alpha$  subunits by RGS proteins and tolerance to opioids like morphine develops. Future efforts to study these phenomena should focus on fitting additional pieces into this puzzle in order to fully define the mechanism underlying the desensitization of MORs in neural cells.

**Keywords:** Mu-opioid receptor, receptor tolerance, nervous tissue, NMDA receptor, protein kinase C, protein kinase A, calcium-calmodulin dependent kinase II, regulators of G-protein signalling proteins.

## 1. INTRODUCTION

Whereas opioids are among the most effective analgesics known, their use is unfortunately restricted by their highly addictive nature. Repeated administration of these drugs brings about a progressive decrease in their potency, and this tolerance implies that higher doses of the opioid are required to obtain the expected outcome with the risk of exacerbating the adverse effects such as respiratory depression and cognitive changes, as well as increasing vulnerability to drug dependence. In animals, tolerance to the antinociceptive effects of opioids can be observed even after a single dose [1,2], and long-term administration not only leads to a profound tolerance but also to physical dependence on these substances. It has been proposed that the mechanisms responsible for acute tolerance parallel those underlying chronic morphine tolerance, because both situations can be modulated by the same agents [3]. In fact, tolerance to single opioid doses is accompanied by some degree of physical dependence, albeit milder than that observed in chronic morphine-dependency [4, 5]. It seems that opioid-induced adaptations after drug administration occur at different levels in the nervous system, beginning with the regulation of opioid receptors themselves, and extending to a complex array of direct and indirect modifications of “downstream” signalling. Functional and gene expression studies have revealed a number of target proteins

that are likely to be involved in *in vivo* adaptation to morphine. As a result, a complex interplay of events at the single cell level and in neuronal networks are thought to contribute to opioid tolerance, including modifications of opioidergic and of other neurotransmitter pathways. Moreover, distinct mechanisms will be more important at different times during opioid chronic exposure.

Morphine initiates its characteristic behavioural responses by activating the Mu-opioid receptor (MOR). This receptor is a G-protein-coupled receptor (GPCR) that regulates diverse effectors, including inwardly rectifying potassium channels, voltage-activated calcium ( $Ca^{2+}$ ) channels, adenylyl cyclase (AC), phospholipases (PLC), etc. Agonist-induced receptor internalization leads to a decrease in the number of functional binding sites and has been associated with the development of tolerance [6]. In fact, the phosphorylation-dependent desensitization by different kinases represents the classical model of GPCR desensitization [7, 8], and it was considered the predominant mechanism mediating the attenuation of GPCR signalling. Interestingly, in cultured cells and in the mature nervous system morphine provokes little or no internalization of MORs, although this does not prevent this opioid from inducing strong antinociceptive tolerance. Conversely, agonists like [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly-ol<sup>5</sup>]-enkephalin (DAMGO), which promotes efficient phosphorylation and endocytosis of MORs, produce only weak tolerance. This is because most of the DAMGO-internalized MORs recycle back to the membrane and resensitize the cell to subsequent exposure to the opioid agonist [9, 10]. Given that most of the internalized MORs elude de-

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struction in the lysosomal compartment, this opioid receptor endocytosis is now conceived as a resensitization step rather than a desensitization step (see references in [11]). Thus, morphine induces a high degree of tolerance because it provokes little phosphorylation and internalization of the MORs [9]. This model of GPCR desensitization is now beginning to be extended beyond the receptor phosphorylation and arrestin binding. It is now well accepted that neural cells have developed specific mechanisms to control MOR activity when the agonists are poor inducers of receptor internalization. Thus, in the nervous system tolerance to MOR-binding agonists involves cellular and molecular adaptive processes including receptor uncoupling from G-proteins [12-16], as well as the regulation of the  $G\alpha$  subunits and of the free  $G\beta\gamma$  dimers generated by the action of morphine on the MORs [17-21].

This review will focus on some of the pharmacological approaches that have consistently been described effective in attenuating or reversing morphine antinociceptive tolerance. These approaches have been selected as candidates to unveil distinct elements within the same regulatory machinery that operate in the post-synaptic membrane. The existing data indicates that the glutamate N-methyl-D-aspartate acid receptor (NMDAR) plays a pivotal role in the desensitization of MORs by morphine. The identification of the integrated pathway connecting MOR activation with the NMDAR-mediated negative feedback loop that depresses MOR signaling will have a profound impact on designing therapeutic interventions for opioid tolerance and dependence.

## 2. ANTI-OPIOID SYSTEMS IN THE REGULATION OF MORPHINE TOLERANCE

### 2.1. The N-Methyl-D-Aspartate/Nitric Oxide Cascade

There is strong evidence that opioid tolerance can be directly modulated by the N-methyl-D-aspartate/nitric oxide cascade [22-25]. In fact, antagonists of NMDAR and inhibitors of the neuronal and endothelial constitutive isoforms of nitric oxide synthase (NOS), rather than the inducible form of NOS, can attenuate opioid tolerance and dependence [26, 27]. Other candidates to produce similar effects include neurotransmitters, peptides such as cholecystokinin, neuropeptide FF, nociceptin, and certain hormones (Table 1).

Glutamate is the major excitatory neurotransmitter in the central nervous system it activates both ionotropic and metabotropic receptor families. Ionotropic receptors directly gate ion channels and they are divided into three major subclasses:  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, and NMDA. Among these, NMDARs have received particular attention because of their crucial roles in excitatory synaptic transmission, plasticity, and neurodegeneration [28]. NMDARs display a number of unique properties that distinguish them from other ligand-gated ion channels. First, the receptor controls a cation channel that is highly permeable to monovalent ions and  $Ca^{2+}$ . Second, simultaneous binding of glutamate and the glycine co-agonist is required for efficient activation of NMDAR. Third, at resting membrane potential the NMDAR channels are blocked by extracellular magnesium and they only open on simultaneous depolarization and agonist binding. Native NMDARs are composed of NR1, NR2 (A, B, C, and D) and NR3 (A and B) subunits. The functional NMDAR is a tetramer inte-

grated by a pair of NR1 subunits associated with at least one type of the NR2/3 subunits [29]. Activation of NMDAR increases intracellular  $Ca^{2+}$  in the postsynaptic neuron and  $Ca^{2+}$  binds to calmodulin (CaM) and activates NOS, stimulating the formation of NO [30]. Production of NO leads to greater glutamate release in surrounding neurons [31], increasing the activity of NMDA and glutamate receptors.

Co-administration of morphine with MK801, a non-competitive NMDAR antagonist, was initially shown to prevent the development of morphine tolerance in rats, mice and guinea pigs [25, 32-34]. Following these initial studies, a large number of NMDA receptor antagonists have been shown to produce similar effects, including non-competitive NMDAR antagonists (dextromethorphan, ketamine, phencyclidine), competitive NMDAR antagonists (LY274614, NPC17742, LY235959), partial glycine agonists (ACPC), glycine antagonists (ACEA-1328), and NOS inhibitors (L-NNA, L-NMMA). Whereas systemic administration of MK801 or LY235959 prevented the development of antinociceptive tolerance to intracerebroventricular (icv) or subcutaneous (sc) morphine, neither of these NMDA antagonists reduced the development of antinociceptive tolerance to fentanyl or DAMGO [35]. However, the development of the tolerance generated by repeated intrathecal (it) administration of DAMGO could be prevented by the co-administration of MK801 [23].

NMDARs have been localized in both supraspinal and spinal structures of mice, rats, and human. At the supraspinal level they have been found in the hippocampus, cerebral cortex, thalamus, striatum, cerebellum, and brainstem [36-40]. At the spinal level, NMDAR has been demonstrated within the substantia gelatinosa of the dorsal horn with a limited presence elsewhere in the spinal grey matter [41]. The distribution of MOR and NMDAR is closely related in many CNS regions and indeed, they co-localize in: spiny neurons within patches of the caudate-putamen nucleus [42]; in the spinal cord dorsal horn particularly within lamina II; in presynaptic and postsynaptic sites in the shells of nucleus accumbens [43]; and in neurons of the nucleus of the solitary tract [44]. In addition, the periaqueductal grey matter (PAG) is densely innervated by glutamatergic projections from the forebrain and MORs also co-localize with NMDARs on PAG neurons [45, 46]. Therefore, it is possible that MORs associate with the NMDAR signalling complex and that a reciprocal regulation exists. Synaptic NMDARs, together with AMPA receptors, are selectively targeted to the postsynaptic side of glutamatergic synapses, where they are structurally organized (and spatially restricted) into large macromolecular signalling complexes composed of scaffolding and adaptor proteins. These structures physically link the receptors to kinases, phosphoprotein phosphatases, and other GPCRs and signalling molecules [47, 48].

Phosphorylation of multiple sites in the cytoplasmic C terminus of the NR1 and NR2 subunits modulates NMDAR activity and affects synaptic transmission. The NMDAR subunit NR2B is the main postsynaptic tyrosine-phosphorylated protein [49]. NR2A is also phosphorylated on tyrosine [50], whereas the NR1 subunit is phosphorylated by protein kinase A (PKA) on serine 890 and 897, and by PKC on serine 896 [51]. The NMDAR currents are governed by a balance between tyrosine phosphorylation and dephospho-

**Table 1.**

Transmitter System/(Drug)	Species/Tolerance Induction	Ref.
Melanotrophin release inhibiting factor (MIF)	rat/chronic	[194]
Oxytocin	mouse/acute	[195]
Cholecystokinin antagonists (L364,718)	rat/acute	[196]
Cholecystokinin octapeptide (CCK8)	mouse/chronic	[197]
Calcium Channel antagonists (nifedipine, verapamil)	mouse/acute	[198]
Dopamine agonists (Bromocriptine)	mouse/chronic	[199]
Delta opioid receptor antagonists (NTI; NTII)	mouse/chronic	[169]
Delta opioid receptor antagonists (TIPP)	rat/chronic	[170]
Delta opioide receptor knock-out	mouse/chronic	[172]
NMDAR antagonists (MK-801)	rat/chronic	[25]
NMDAR antagonists (LY235959)	mouse/acute	[35]
Neuropeptide FF	rat/chronic	[200]
NO inhibitors (NG-nitro-L-arginine)	mouse/chronic	[26]
5-HT receptor antagonists (ritaserin)	mouse/chronic	[201]
Calcitonin gene-related peptide	mouse/acute	[202]
Imidazoline agonists (agmatine)	mouse/chronic	[203]
Substance P metabolites (SP1-17)	mouse/acute	[204]
Neurosteroids (allopregnanolone)	mouse/chronic	[205]
Melatonin	mouse/chronic	[206]
Nociceptin	mouse/chronic	[207]
Preproenkephalin knock-out	mouse/chronic	[185]

Inhibition of the development of tolerance to the analgesic effects of opioids by compounds acting on different neurotransmitter systems. A single (acute) or multiple (chronic) doses of the drug were administered to induce tolerance in rodents.

phorylation [52], and protein tyrosine kinase Src can mediate this up regulation [53]. Src is the principal member of a family of non-receptor tyrosine-kinases (NRTKs) with nine members. Five of the NRTKs are expressed in the mammalian CNS (Src, Fyn, Yes, Lck and Lyn) [54], and all but Lck have been shown to be components of the NMDAR complex in the brain and spinal cord. Inhibition of PKC does not alter the Src-induced enhancement of NMDAR responses, whereas inhibition of Src depresses the potentiation of NMDAR currents by activated PKC [55]. Indeed, PKC does not up regulate NMDARs in neurons from Src<sup>-/-</sup> mice. Interestingly, opioids might also modulate NMDA receptor function through the activation of NRTKs [56]. Thus, the activation of GPCRs seems to be coupled to NR2B tyrosine-phosphorylation and requires PKC, intracellular Ca<sup>2+</sup> release, and Src activation [57, 58].

## 2.2. Protein Kinases and Phosphatases in MOR Desensitization

Phosphorylation of GPCRs by protein kinases has been linked to receptor uncoupling and internalization, which affects cellular desensitization and resensitization [59]. Pharmacological interventions at one or more of the protein kinases, PKC, PKA, CaMKII, G-protein coupled receptor kinases (GRK), and phosphatases have provided valuable evidence about the essential role of phosphorylation in pro-

moting and maintaining opioid tolerance [8] (Table 2). At the receptor level, MOR phosphorylation augments after exposure to opioid agonists in a Ca<sup>2+</sup>-dependent manner [60], and it is required for cellular desensitization [61-65]. Depending on the model, it is possible that different kinases/mechanisms are responsible for the development of tolerance (e.g., acute vs chronic tolerance, cellular vs rodent models). Furthermore, different opioids have been shown to display different efficacy in perpetuating receptor-mediated internalization [66, 67], a process that is intimately linked to the action of one or more kinases. Therefore, results from such studies have to be interpreted in the context of the specific model system and opioid drug used. Caution should therefore be exercised when extrapolating the findings beyond these cellular systems, especially in view of the fact that cell lines do not always express signalling machinery comparable to that of neural cells [68, 69].

### 2.2.1. Protein Kinase C

The translocation of PKC from the cytosol to the plasma membrane is considered an indicator of PKC activation [70]. Opioid tolerance has been reversed and/or prevented in various animal models using PKC inhibitors [4, 71-78], suggesting a critical role for PKC in this process (Table 2). Indeed, membrane-bound PKC appears to increase after chronic administration of opioids, yet it is still controversial how per-

Table 2.

Protein Kinase/(Inhibitor)	Species/Tolerance Induction	Ref.
PKA		
KT-5720	mouse/chronic	[73-75,94]
4-cyano-3-methylisoquinoline	mouse/chronic	[75]
Knock-down alpha catalytic subunit of mouse PKA	mouse/chronic	[93]
PKC		
H7	rat/chronic	[78]
H7	mouse/acute	[4]
Chelerythrine	rat/chronic	[71]
Calphostin C	mouse/acute	[77]
Gö-6976 and bisindolylmaleimide I	mouse/chronic	[75]
Gö-7874 and sangivamycin	mouse/chronic	[74,76]
PKC $\alpha$ , $\gamma$ and $\epsilon$ peptide inhibitors	mouse/acute	[85]
Knock-out PKC $\gamma$	mouse/chronic	[82]
Knock-down PKC $\gamma$	rat/chronic	[83]
CaMKII		
KN62, KN93	rat/chronic	[99]
KN93	mouse/chronic	[159]
knock-down CaMKII	rat/chronic	[99]

Inhibition of the development of tolerance to the analgesic effects of opioids by Protein Kinase inhibitors. A single (acute) or multiple (chronic) doses of the drug were administered to induce tolerance in rodents.

sistent PKC activation is achieved in opioid tolerance because one would expect the kinase to desensitize after prolonged activation. One plausible mechanism may be through the interaction of PKC with the NMDAR. Phosphorylation of NMDAR by PKC has been shown to enhance NMDAR function [51]. Therefore, PKC activated as a result of opioid activation could potentially increase the activity of the NMDA receptors. The activated NMDAR augments Ca<sup>2+</sup> permeation, which may in turn enhance the activation of the PKC. As a result, a positive feed forward loop between PKC and the NMDA receptors may exist in opioid tolerance.

Only a few PKC isoforms seem to be implicated in opioid tolerance. PKC $\gamma$  immunoreactivity increases in dorsal horn of the spinal cord in morphine-tolerant rats [71, 79]. Accordingly, morphine produces significantly less antinociceptive tolerance in mice and rats lacking PKC $\gamma$  [80-82]. PKC $\alpha$  is another isoform that is up-regulated during opioid tolerance [71]. Antisense oligonucleotides targeting spinal cord PKC $\alpha$  can prevent morphine tolerance in rats [83]. Moreover, PKC $\beta$  may also be regulated by opioids, although its role in tolerance is less clear [84]. Recently, through the use of isoform-specific PKC peptide inhibitors it was proposed that PKC $\alpha$ ,  $\gamma$  and  $\epsilon$  are the isoforms responsible for maintaining morphine tolerance in mice [85]. PKC beta (I), beta (II), delta, theta, epsilon, eta and xi appeared to be less relevant in the process.

### 2.2.2. Protein Kinase A

The cAMP-dependent protein kinase or PKA is another important protein kinase that can be modulated by opioid drugs. The information carried by cAMP second messengers and PKA is propagated by a number of downstream effectors

including the transcription factor CREB (cAMP response element-binding protein) [86]. Opioid-mediated up-regulation of the cAMP pathway and activation of CREB occurs in the locus coeruleus, an area important in physical dependence [87]. Moreover, by increasing NMDAR currents opioids can activate calcium- and calmodulin-regulated ACs and promote PKA activity [88]. In the post-synapse, the interplay between activated p-Thr286 CaMKII and serine/threonine protein phosphatase 1 (PP1) regulates long-term potentiation [89]. The PP1 dephosphorylates and inactivates CaMKII when calcium is lowered to a basal level [90] and by inhibiting the action of PP1, PKA prolongs the activity of the CaMKII here [91, 92]. Initially, targeted inhibition of PKA produced mixed results on opioid tolerance *in vivo*, probably due to the poor selectivity of the kinase inhibitors or the different degrees of opioid antinociceptive tolerance induced by the agonists (Table 2). However, there are an increasing number of reports that consistently show PKA inhibitors to partially [74, 93] or fully [73, 94] block antinociceptive tolerance to opioids.

### 2.2.3. Calcium/Calmodulin-Dependent Kinase II

CaMKII is a Ca<sup>2+</sup>/CaM-activated protein kinase that is highly abundant in the CNS. The Ca<sup>2+</sup> influxes *via* the NMDARs provoke the activation and autophosphorylation of CaMKII at Thr286 [95]. Anatomically, MORs and CaMKII co-exist in distinct pain-processing brain regions [96] and the co-localization of both the receptor and kinase was interpreted as a means to facilitate MOR desensitization. On the other hand, intracellular Ca<sup>2+</sup>, CaM and CaMKII can all be regulated by opioids and significantly, CaMKII activity is

increased in brain areas of animals rendered tolerant to morphine [97, 98]. Upon the activation of MORs, the potentiation of CaMKII is mediated by an increase in cytosolic  $Ca^{2+}$  and recruitment of CaM to form the  $Ca^{2+}$ /CaM complexes necessary to activate this kinase.

Using *in vivo* models of tolerance and dependence, it has been shown that CaMKII regulates the effects of opioids (Table 2). Thus, intrahippocampal administration of the specific inhibitors KN62 and KN93 in rats significantly reduced morphine tolerance [99, 100]. Furthermore, administration of antisense oligonucleotides that specifically decrease the expression of CaMKII, also attenuates morphine tolerance and dependence [99]. The effects of CaMKII inhibitors do not appear to depend on their ability to interfere with associative learning because acute supraspinal kinase inhibition by icv administration of KN93 effectively reversed acute morphine tolerance in the mouse [101]. Moreover, the effect of KN93 was not due to any direct effect on nociception or antinociception given that KN93 itself did not produce antinociception or interfere with acute morphine antinociception. Indeed, the acute CaMKII inhibition mediated by KN93 reversed the morphine tolerance and dependence already established, suggesting that both spinal and supraspinal CaMKII are essential to maintain opioid tolerance and dependence.

Beyond the MOR and NMDAR, many downstream effectors can be affected by CaMKII, including transcription factors such as CREB [102], known to be involved in morphine addiction. Therefore, it may be possible that inhibition or down-regulation of CaMKII prevents morphine-induced activation of this kinase and the subsequent phosphorylation of opioid receptors and other signal proteins, thereby attenuating tolerance and dependence. Among the multiple proteins implicated in the MOR signalling cascade the long isoform of phosphatidylinositol-3-OH kinase (PI3K) is worth mentioning. PI3K is uniformly distributed throughout the CNS and it is a substrate for CaMKII and casein kinase 2 phosphorylation [18, 103]. PI3K is considered a specific regulator of free  $G\beta\gamma$  dimers [18, 104] and thus, by binding  $G\beta\gamma$  dimers it prevents them from acting on effectors and it obstructs the re-association of trimeric G-proteins. Recent work has revealed that icv administration of morphine induces the activation of CaMKII and impairs the association of MORs with  $G\alpha$  subunits [11]. The inhibition of CaMKII permits the  $G\beta\gamma$  dimers to re-associate with  $G\alpha$  subunits under the control of the MORs and accordingly, no tolerance to morphine develops. These results indicate that CaMKII phosphorylation of PI3K stabilizes the PI3K. $G\beta\gamma$  complex at 14-3-3 proteins, thereby preventing MORs from recovering control over the G proteins [18, 105].

#### 2.2.4. Protein Phosphatases

If phosphorylation by protein kinases is critical to initiate the processes that lead to opioid tolerance, the “off-switch” is provided by the action of PPs. However, the role of PPs in opioid tolerance has not been convincingly determined. It has been shown that okadaic acid, an inhibitor of PP1 and PP2A, enhances the antinociceptive effect of morphine in opioid tolerant mice, suggesting that PP1 and/or 2A may actually contribute to the development of morphine tolerance [106]. Additionally immunosuppressants such as FK506 (tacrolimus) and cyclosporine A, immunophilin ligands that inhibit the  $Ca^{2+}$ /CaM-dependent PP calcineurin (PP2B)

[107], block the development and expression of opioid tolerance [108]. However, these agents also reduce NO production by inhibiting dephosphorylation of nNOS. Therefore, a major part of their effects may be due to the inhibition of NO/cGMP/PKG or other pathways. More direct approaches by activating PPs have so far not been tested experimentally.

### 2.3. RGS Proteins on Opioid Tolerance

A current area of much interest in opioid tolerance research is the group of RGS signalling proteins. The RGS proteins act as GTPase-activating proteins (GAP) that rapidly terminate receptor-activated  $G\alpha$ GTP signaling by accelerating the hydrolysis of GTP to GDP. It is now accepted that the potency and duration of the effects initiated at GPCRs depends on the continuous and efficient reconstitution of the pool of receptor-regulated heterotrimeric G proteins [12, 13]. Thus, RGS proteins help synchronize the presence of receptor agonists with the regulation of their target effectors. There are approximately 30 RGS proteins that mostly act as negative modulators of GPCR-mediated signalling [109]. They do so by acting as GAPs to accelerate  $G\alpha$ GTP inactivation *via* stimulation of  $G\alpha$ -GTPase activity [109], and if this is achieved before the  $G\alpha$ GTP reaches and regulates the effectors, then the RGS antagonise G protein effectors. Alternatively, they may exert their effects by sequestering  $G\alpha$  subunits, preventing them from reconstituting the trimeric  $G\alpha\beta\gamma$  proteins in a manner regulated by the GPCRs [19].

The influence of the RGS proteins on MOR signalling and the decline in opioid efficacy has been demonstrated at both cellular and systems levels. In neurons of the midbrain PAG, the MORs associate with a series of RGS proteins, including RGS4, members of the RGS-R7 and Rz subfamilies, and RGS14 [110]. RGS4 interacts directly with the C terminus of MOR [111, 112], and members of the RGS-R7 subfamily seem to bind to this region of the MOR through their N-terminal DEP domain [112, 113]. Furthermore, the protein kinase C-interacting protein (PKCI) interacts with both the C terminus of the MOR [114] and the RGSZ1 and RGSZ2 proteins coupled to MORs through their N-terminal cysteine rich domain. This domain binds to the conserved triad of histidines at the C terminus of the PKCI in a zinc-dependent manner [115] and suggests that a stable link can be established between the MOR and these regulatory proteins in the CNS. Functional studies have demonstrated that different RGS subtypes exert diverging effects on the regulation of opioid analgesia and the development of tolerance to morphine and reviews that have analyzed the specific regulation exerted by RGS protein subtypes are now available [19, 110]. In brief, it is clear that the RGS proteins control the activity of MORs in the CNS through both GAP-dependent (RGS-R4 and RGS-Rz) as well as by GAP-independent mechanisms (RGS-R7).

#### 2.3.1. The R4 Subfamily

RGS2, RGS3, RGS4, and RGS8 are all members of the R4 subfamily and they regulate MOR function in certain neurons and tissues, presumably as a result of the GAP activities of their RGS domain and thanks to their spatial and temporal coexpression with opioid receptors [17, 19, 110-112]. The expression of RGS2 and RGS4 in cultured cells significantly attenuates the inhibition of AC exerted by the

MOR agonists morphine and DAMGO [116]. In mice, the RGS4, RGS8 and RGS16 proteins all negatively modulate analgesia because reducing their expression augmented the effects of MOR- and DOR-binding agonists. RGS4, RGS8 and RGS16 can modulate the activity of morphine, and RGS4 also influences that of DAMGO, and of the DOR agonists [D-Pen<sup>2,5</sup>]-encephalin (DPDPE) and [D-Ala<sup>2</sup>]deltorphin II [17]. These results can be interpreted in terms of a prolonged effect of the activated G $\alpha$ GTP subunits on their target effectors and hence, the increase in the activity of opioid agonists. However, the dampening of the effects of agonists when the levels of the RGS2 and RGS3 proteins are reduced following antisense administration indicate that these RGS proteins augment the effects of opioids by a mechanism independent of their GAP function. RGS2 and RGS3 interact with AC and negatively regulate the production of cAMP [19]. Significantly, the knockdown of the RGS-R4 members does not prevent the appearance of tolerance to morphine [17, 19].

### 2.3.2. The R7 Subfamily

Blocking the expression of RGS9-2 or G $\beta$ 5 with specific antisense oligonucleotides greatly enhances the potency and duration of the antinociceptive effects produced by morphine and DAMGO. Such treatments also successfully prevent acute morphine tolerance [17, 20, 117, 118]. Moreover, in a cell culture system of mouse hippocampal neuroblastoma cells, suppression of RGS9 expression by antisense oligonucleotide significantly inhibits chronic morphine-induced up-regulation of AC activity and partially reverses the chronic morphine effect on abolishing DAMGO-induced GTP $\gamma$ S high-affinity binding [119]. Through the similar use of antisense technology, RGS6, RGS7, and RGS11 (members of the same R7 subfamily) were all shown to negatively regulate morphine activity [117]. During chronic morphine treatment RGS7, RGS9-2, and RGS11 mRNA levels increase in most regions of the brain, especially in the striatum and PAG, and their protein expression also augments after chronic but not acute morphine administration [120]. The absence of acute opioid tolerance when RGS proteins of R7 subfamily are silenced in mice is thought to be mostly due to their direct role in the long-term sequestering of MOR-activated G $\alpha$  subunits [17, 21, 117, 118]. This transfer (sequestering) of G $\alpha$  subunits occurs when the effects of morphine reach a certain level and it is facilitated by post-translational modification of these RGS proteins. Such modification permits them to bind to the activated G $\alpha$  subunits but precludes their GAP activity on them or reduces their capacity to dissociate from the RGS domain. Among such modifications, the phosphorylation of serine residues in the RGS domain of RGS-R7 proteins and the ensuing binding of 14-3-3 proteins appear to be particularly relevant. The consolidation of this transfer is time-dependent and could involve the activation of glutamate NMDAR, probably *via* PKC [21, 121]. In accordance with this model, when exogenous G $\alpha$  subunits are made available to MORs the duration of opioid-evoked analgesia extends and no tolerance develops [13]. The negative regulation of G-protein signalling by R7 proteins could also account for the long duration of acute opioid tolerance that persists for several days until the initial response to the opioids is recovered [18].

### 2.3.3. The Rz Subfamily

The knockdown of neural members of the RGS-Rz subfamily, RGS17(Z2), RGS19(GAIP) and RGS20(Z1), does not affect the activity of DOR agonists. The RGS-Rz proteins are selectively linked to MORs and they reduce the amplitude and duration of morphine and DAMGO analgesia [20, 110, 122, 123]. When RGSZ1 expression is impaired, the analgesia produced by morphine is enhanced and its effects are notably prolonged [123]. In contrast, knockdown of the RGSZ2 protein promotes an initial sharp increase in analgesia but also results in the rapid desensitization of some of the target effectors [20, 122]. RGS proteins of the Rz subfamily also retain activated G $\alpha$  subunits, and this raises the possibility that RGS-R7 and RGS-Rz act in concert to achieve this effect. Upon Mu-opioid receptor activation, there is a strong increase in the association of G $\alpha$  subunits with RGSZ2 proteins, which persists for more than 24 h [11, 122, 124]. Only a moderate increase was observed with RGSZ1. Interestingly sumoylated forms of these RGS are found associated with MORs [124]. Furthermore, Gai2 and G $\alpha$ z subunits co-precipitate with the sumoylated forms of RGSZ1/Z2 proteins, but to a lesser extent with the Ser phosphorylated SUMO-free form of RGSZ1. Therefore, sumoylation regulates the biological activity of RGS-Rz proteins and it is likely that it serves to switch their behaviour from that of a GAP for activated G $\alpha$ GTP subunits to that of a scaffold protein for specific signalling proteins.

### 2.3.4. The R12 Subfamily

RGS14, a member of the R12 subfamily of RGS proteins, is a multifaceted regulator of signal transduction. A recent report showed that on removal of RGS14, morphine becomes capable of stimulating the phosphorylation and internalization of neural MORs [121]. In these conditions, morphine produces much less activation of CaMKII and less acute tolerance. Thus, morphine triggers a NMDAR/CaMKII-mediated mechanism to desensitize the MORs in the plasma membrane and receptor phosphorylation/internalization disrupts this negative feedback regulation. We found that when RGS14 expression was reduced in mice, morphine promotes Serine 375 phosphorylation in the C terminus of MORs, as well as the internalization and recycling of these receptors. These mice displayed an increased response to morphine antinociception and the analgesic tolerance to this opioid developed at more slowly rate.

## 3. CROSS-REGULATION BETWEEN MOR AND NMDAR

It is obvious that there has been a prolific advance in our understanding of the molecular mechanisms underlying opioid tolerance. The results from many laboratories highlight that neurons in particular have their own means to control GPCR function, and that a few signalling proteins are critical in this process. Notably, the expression of NMDARs, certain RGS proteins such as RGSZ1, RGSZ2, members of the RGS-R7 subfamily, and of G $\alpha$ z subunits, is virtually restricted to nervous tissue, and these proteins certainly influence the regulation of neural MORs. Moreover, the approaches that effectively diminished opioid tolerance could be targeting distinct elements of a common regulatory pathway, and here the NMDARs seem to play a pivotal role. Our current knowledge of this cellular signalling suggests that the

cross-talk between MORs and NMDARs could occur within the same cell, at the same postsynapsis and probably, in the same compartment without ruling out the possibility that direct interactions occur. This regulatory loop would commence when the opioid agonist activates the MORs and their signals are recognised by the NMDARs to increase the  $\text{Ca}^{2+}$  currents. The activation of the NMDARs will in turn negatively influence the stamina of MOR signalling leading to opioid desensitization. Several observations sustain this model, including the fact that: in different neural areas both MORs and NMDARs co-localize in the postsynapsis; positive regulators of NMDARs such as PKC and Src are activated by GPCR agonists; the CaMKII, and probably PKA as well, that promote MOR tolerance are activated by NMDARs; the negative regulator of MOR function, PhLPI, sequesters MOR-generated  $\text{G}\beta\gamma$  dimers in a CaMKII-dependent manner, and makes the sequestering of  $\text{G}\alpha$  subunits by RGS-R7 and RGS-Rz proteins possible.

The following section analyzes the more relevant points of this proposed cross-regulation between MOR and NMDAR in greater detail.

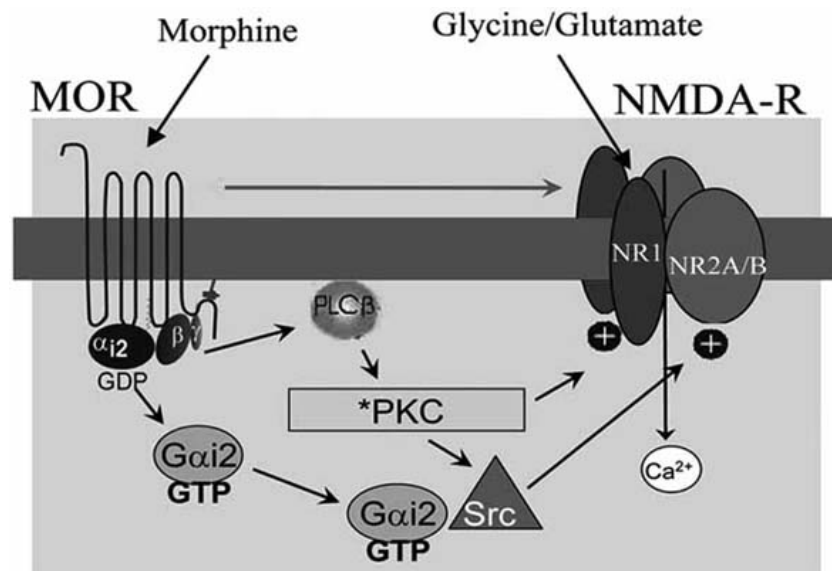
### 3.1. Regulation of NMDARs by Activation of MORs

The MOR is known to potentiate NMDAR-mediated glutamate responses through the activation of PKC [125]. In general, GPCRs regulate the activity of NMDARs by means of NTRKs such as Src and Fyn, and also through Ser/Thr kinases like PKC [56]. Src and Fyn phosphorylate specific tyrosine residues in the cytosolic tail of NR2A, NR2B and probably NR3A/B subunits as well [53], producing an increase in the permeation of  $\text{Ca}^{2+}$  ions towards the cytosolic side of the postsynapsis. PKC and PKA also contribute to the activation of the NMDARs probably acting upstream of NTRKs and enhancing their activity on these receptors [55, 56, 126]. The PKC and PKA could also regulate NMDAR function by other means [127, 128], for example through the phosphorylation of serine and threonine residues in the cyto-

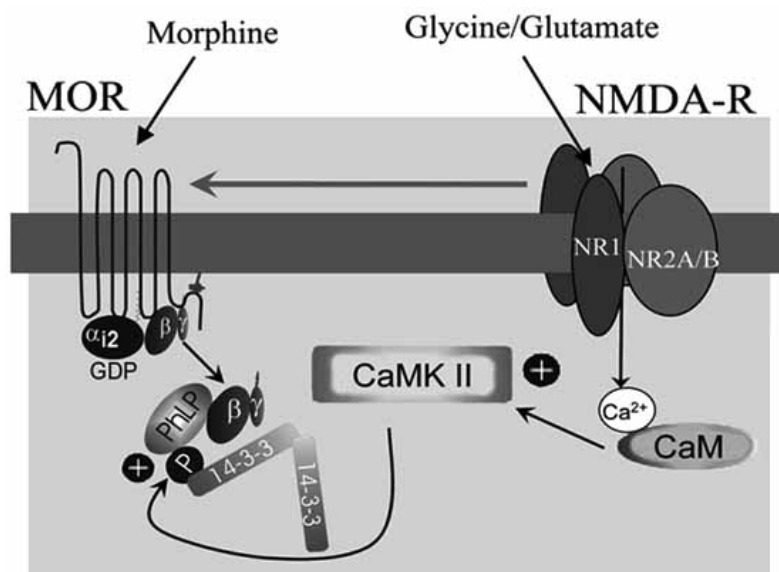
solic regions of NR1 and also of NR2 subunits [51,129, 130].

Src and PKC can be regulated by signalling proteins that are activated by the MOR (Fig. 1). Indeed, agonists of MORs generate  $\text{G}\beta\gamma$  dimers that activate  $\text{PLC}\beta$ , which in turn activates the PKC involved in the potentiation of the NMDAR [125]. Given that the kinase activity of Src is enhanced by direct binding with  $\text{G}\alpha\text{iGTP}$  subunits [131] it is possible that GPCRs [132, 133], and particularly MORs, promote the activation of Src kinases associated with the NMDAR complex [134]. Thus, Src and PKC collaborate to carry the signals from GPCRs to NMDARs, and display a synergistic convergence although the strength of the NMDAR response seems to depend on the relative time of arrival of the two kinases. Indeed, the prior action of Src (probably activated by a PKC pathway) on NR2/3 subunits seems to be required for PKC to produce the direct potentiation of NMDAR-mediated  $\text{Ca}^{2+}$  permeation.

The selectivity of many kinases is principally achieved by their translocation to the environment of the appropriate target proteins [135]. Src is already associated with the NMDAR complex and therefore it can be rapidly activated by MORs *via* PKC and  $\text{G}\alpha\text{iGTP}$  subunits. Activated Src potentiates NMDAR currents by removing the tonic inhibition caused by extracellular zinc [136]. The result is an increase in the entry of  $\text{Ca}^{2+}$  which contributes to the translocation of PKC to potentiate the activity of NMDARs [128,137]. This process is similar to the potentiation of NMDARs mediated by metabotropic glutamate receptors, which also involves a rise in intercellular  $\text{Ca}^{2+}$  and activation of PKC [138, 139]. The  $\text{Ca}^{2+}$  flux through the NMDARs recruits CaM and promotes the formation of the  $\text{Ca}^{2+}$ -CaM complexes necessary to propagate the signals originated by glutamate/glycine at the NMDARs (Fig. 2). In the postsynaptic densities, inactive PKCs are associated to A-kinase anchoring proteins (AKAPs) and the  $\text{Ca}^{2+}$ -CaM complex disrupts this interac-



**Fig. (1). MOR-mediated potentiation of NMDAR currents.** Agonist-activated MORs propagate their signals through  $\text{G}\alpha\text{GTP}$  subunits and  $\text{G}\beta\gamma$  dimers. The  $\text{G}\alpha\text{i2GTP}$  subunits bind to and activate NTRK such as Src to phosphorylate specific tyrosine residues of the cytosolic C terminus of NR2A/B subunits. This foments the permeation of extracellular  $\text{Ca}^{2+}$  ions towards the cytosolic side of the post-synapses. The  $\text{G}\beta\gamma$  dimers bind to and activate phospholipases, which in turn activate PKC. PKC may act up-stream of Src to potentiate NMDARs currents or they may also act directly on NR1 subunits.



**Fig. (2).** NMDARs exert a negative feedback on MOR function by activating CaMKII. The increased levels of cytosolic  $\text{Ca}^{2+}$  ions recruit CaM from protein stores and promoted the formation of  $\text{Ca}^{2+}$ -CaM complexes that are able to regulate a variety of proteins at synapses. CaMKII is activated and translocated to the MOR environment where it phosphorylates the PhLP. PhLP binds competitively to opioid-generated free  $\text{G}\beta\gamma$  dimers and the action of CaMKII enables the P-Ser PhLP. $\text{G}\beta\gamma$  complex to bind to the phosphoserine-binding protein 14-3-3. The outcome of this process is the sequestering of a fraction of the  $\text{G}\beta\gamma$  dimers that the MORs require to efficiently reconstitute the trimeric  $\text{G}\alpha\beta\gamma$  proteins that are under their control. Accordingly, the MORs are desensitized.

tion [140]. This has been documented for the neuron-specific  $\text{PKC}\gamma$  which is clearly implicated in the desensitization of MORs [81, 82]. Thus, the sequence of events would be as follows: the Src-evoked increase in  $\text{Ca}^{2+}$  recruits CaM from a series of CaM-binding proteins named calpacitins [141], such as neurogranin, neuromodulin and probably from the MOR as well [142-144]; the  $\text{Ca}^{2+}$ -CaM complexes displace the inactive  $\text{PKC}\gamma$  from AKAP79 then increasing its translocation to the membrane [145];  $\text{Ca}^{2+}$ -CaM binds  $\text{PLC}\beta$  [146] to potentiate the MOR-induced  $\text{G}\beta\gamma$  activation of the phospholipase [147] and thus, increases the activation of the  $\text{PKC}$  in a  $\text{Ca}^{2+}$  and diacylglycerol-dependent manner. In this way, the activation of MORs could cause the positive regulation of NMDARs by a two step mechanism that requires the initial activation of Src, probably *via*  $\text{PKC}$ , followed by the translocation of active  $\text{PKC}$  near the NMDARs (Fig. 1).

There is some variation in the subunit composition of the NMDARs in different neural areas, as well as during the development and maturation of the CNS. Whereas NR1 is ubiquitously expressed in nervous tissue [148], there is a pronounced regional heterogeneity in the expression of the NR2 subunits, which are also regulated during development [149]. Given the plasticity of the NMDARs, it would be expected that the activation of MORs might bring about important changes in the density and subunit composition of these glutamatergic receptors in the membrane. In fact, changes in the expression of NMDARs after prolonged treatment with opioids have been shown [22,150] as well as in the NMDAR-mediated excitatory postsynaptic currents (EPSCs) [151]. Moreover, chronic morphine administration brings about region specific changes in the expression of NMDAR subunits, a process that could influence the excitability and integrative properties of the synapses [152-154]. Thus, the development of tolerance to MORs may also promote pro-

found changes in the function of the opioid-regulated NMDARs. Future research should focus on the elements that govern the cross-regulation of MOR-NMDAR in the postsynapse.

### 3.2. PKC and PKA Facilitate the NMDAR-Mediated Activation of CaMKII and NOS

Activation of the MOR enhances NMDAR  $\text{Ca}^{2+}$  currents in the postsynapsis, as well as the formation of  $\text{Ca}^{2+}$ -CaM complexes that regulate diverse signalling proteins. Among these, CaMKII, NOS, AC-PKA and GPCR-released  $\text{G}\beta\gamma$  dimers, contribute to the development of tolerance to morphine antinociception [99, 100, 105, 155]. The activation of NOS stimulates the formation of NO [30] and leads to greater release of glutamate in surrounding neurons [31], thereby increasing the activity of NMDA and other glutamate receptors. Thus, NOS contributes to MOR desensitization *via* potentiation of NMDARs. The mechanism by which CaMKII, AC-PKA and MOR-released  $\text{G}\beta\gamma$  dimers promote opioid tolerance is distinct and will be discussed later.

Interestingly,  $\text{PKC}$  is also required to keep CaMKII activated. As mentioned previously, free levels of CaM are regulated by its binding to a series of proteins and sharp increases in local  $\text{Ca}^{2+}$  releases the CaM from these stores, enabling it to form  $\text{Ca}^{2+}$ -CaM complexes. The activated  $\text{PKC}$ s phosphorylate calpacitins at the CaM binding locus and prevents the re-binding of this protein, making the formation of  $\text{Ca}^{2+}$ -CaM complexes possible even in the presence of low levels of  $\text{Ca}^{2+}$  [see e.g. 127]. Thus,  $\text{PKC}$  would help to maintain the levels of  $\text{Ca}^{2+}$ -CaM complexes higher than expected from the cytosolic levels of  $\text{Ca}^{2+}$ . Notably, the activated  $\text{PKC}$  also increases the efficiency of NMDAR signalling. The open probability of NMDAR channels is greatly reduced by the binding of the  $\text{Ca}^{2+}$ -CaM complex to the C1 region of NR1

subunits. PKC phosphorylates serine residues in this C1 region and prevents the binding of  $\text{Ca}^{2+}$ -CaM, thereby increasing the open probability of the channel. Activated PKC also increases NMDAR signalling by phosphorylating the site of  $\text{Ca}^{2+}$ -CaM binding on the PMCA2a  $\text{Ca}^{2+}$  pump in neuronal cell membranes. The binding of the complex to the pump stimulates the removal of cytosolic  $\text{Ca}^{2+}$  [see 127]. Thus, the positive effect of PKC on NMDAR is not just mediated by NTRKs but PKC also directly affects NR1 subunits and CaM-binding proteins. Together, these actions of PKC clearly contribute to the duration of CaMKII activation in the postsynapsis. Moreover, the PKC-mediated activation of NMDAR also enhances the activation of the PKC. The increase in the permeation of  $\text{Ca}^{2+}$  and the binding of  $\text{Ca}^{2+}$ -CaM to PLC $\beta$  [146, 147] potentiates PKC in a  $\text{Ca}^{2+}$  and diacylglycerol-dependent manner. This positive feedback loop between PKC and the NMDA receptors could contribute to maintain opioid tolerance after the effects of the opioid agonist have ceased.

In the postsynapse, PKA also helps keep CaMKII active. The transient entry of calcium through the NMDAR channel stimulates the autophosphorylation of CaMKII at Thr286. This CaMKII isoform binds CaM with high affinity and then maintains its activity in a calcium-independent manner [89]. PKA becomes activated at high calcium concentrations [88] and phosphorylates the regulatory protein inhibitor-1, which in turn inhibits the PP1 responsible for dephosphorylating p-Thr86 CaMKII [91, 92]. This inhibition of PP1 helps to maintain the long-term potentiation of the post-synapse. When the calcium concentration falls to basal levels, the activity of AC-PKA diminishes and CaMKII is dephosphorylated by PP1 at p-Thr286, inactivating the kinase at low levels of calcium [90]. Thus, PKA favours the activity of CaMKII particularly in circumstances of high calcium entry through NMDARs, and therefore, it could also participate in the development of tolerance to MOR-binding agonists.

### 3.3. CaMKII Supports the Sequestering of MOR-Activated G $\beta\gamma$ Dimers by Phosphorylation of Glycosylated PhLPI

The long isoform of the phosducin-like protein regulates the activity of G $\beta\gamma$  dimers. In the nervous system, PhLPI exists as both a 38 kDa non-glycosylated isoform and as glycosylated isoforms of about 45, 100 and 150 kDa. After the activation of MORs, activated CaMKII is translocated close to the MORs where it phosphorylates the PhLPI proteins. This phosphorylation augments the association of glycosylated PhLPI isoforms with free G $\beta\gamma$  dimers and phosphoserine-binding 14-3-3 proteins (Fig. 2). Seeing that the PhLPI.G $\beta\gamma$  complex can be disrupted by third party proteins, e.g. G $\alpha$ GDP subunits to re-form trimeric G $\alpha\beta\gamma$  under control of MORs, the CaMKII phosphorylation of PhLPI stabilizes the PhLPI.G $\beta\gamma$  complex by promoting its binding to 14-3-3 proteins [18,105]. The binding of  $\text{Ca}^{2+}$ -CaM generated by the MOR-induced activation of NMDARs to free G $\beta\gamma$  dimers prevents their re-association with G $\alpha$ GDP subunits but permits their interaction with PLC $\beta$  [144] and probably with PhLPI. PKC $\gamma$  could remove  $\text{Ca}^{2+}$ -CaM from the G $\beta\gamma$  subunits allowing them to bind with G $\alpha$  subunits [156]. All these effects of  $\text{Ca}^{2+}$ -CaM on CaMKII and G $\beta\gamma$  dimers dampen the regulation of G proteins by the MORs and contribute to the development of tolerance to the antinociceptive

effects of morphine. Thus, a common pathway appear to exist in which  $\text{Ca}^{2+}$ -CaM, AC-PKA, activated CaMKII and PhLPI act to desensitize MORs (Fig. 2).

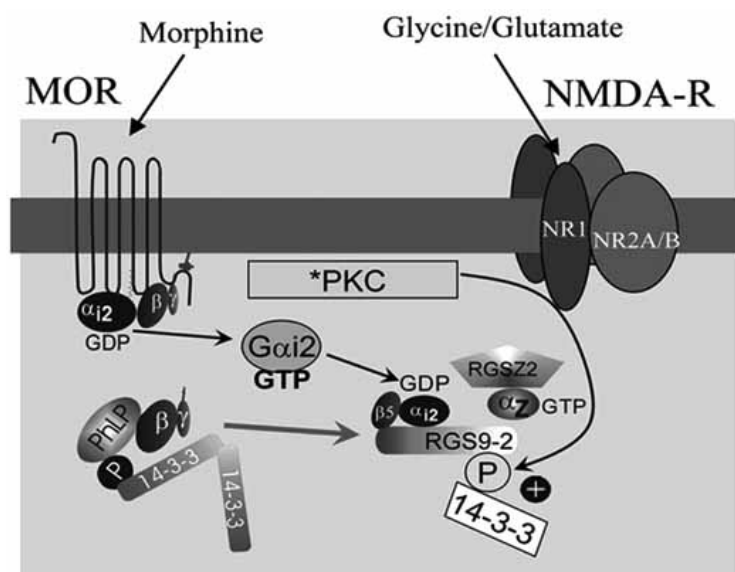
### 3.4. RGS Proteins of R7 and RZ Subfamilies Sequester MOR-Activated G $\alpha$ Subunits

The work of Garzón and colleagues [17] demonstrated the implication of RGS9-2 proteins in the production of MOR desensitization in the CNS, and gave rise to a series of studies that substantiated the role for the entire RGS-R7 protein family in the production of morphine tolerance [20, 21, 110, 117, 118]. Similarly, the participation of some of the RGS-Rz proteins in this process has been demonstrated, particularly that RGS17(Z2) [11, 19, 20, 122-124], emphasising the participation of RGS proteins in morphine-induced tolerance [7, 112, 119, 157].

The proposed sequence of events starts with the need of the opioid-generated free G $\beta\gamma$  dimers to accelerate the release of G $\alpha$ GDP subunits from their selective GTPase activating proteins, the RGS proteins [158, 159]. Therefore, the sequestering of G $\beta\gamma$  subunits by PhLPI.14-3-3 protein complexes facilitates the morphine-induced uncoupling of MORs that occurs in the absence of receptor internalization. Indeed, the reduction in free G $\beta\gamma$  dimers brought about by PhLPI increases the loss of receptor-activated G $\alpha$  subunits in the environment of the MORs [21], which therefore associate with RGS proteins of the R7 and Rz subfamilies [11, 21, 122, 124] (Fig. 3). This persistent interaction seems to be facilitated by post-translational modifications of these RGS proteins, permitting them to bind to the activated G $\alpha$  subunits but precluding their GAP activity on them, or delaying its release. Among such modifications, the phosphorylation of serine residues in the RGS domain of RGS-R7 proteins and their ensuing association with 14-3-3 proteins appears to be particularly relevant [21]. Similarly, the sumoylation of specific sequences in the RGS domain of the RGS-Rz proteins may also participate in these events [124]. Hence, both the retention of G $\beta\gamma$  dimers by PhLPI proteins and that of G $\alpha$  subunits by certain RGS proteins could act together to desensitize the activity of morphine (Fig. 4).

## 4. RECEPTOR OLIGOMERIZATION IN THE REGULATION OF MORPHINE TOLERANCE AND DEPENDENCE: ROLE OF DELTA RECEPTORS

Electrophysiological and immunohistochemical studies have shown that both MORs and DORs colocalize in the same axonal terminals [160,161]. Moreover, both receptors have recently been shown to co-immunoprecipitate from parts of the nervous system known to be involved in pain transmission, such as the spinal cord [162] and PAG [110]. Indeed, divalent opioid ligands can bind simultaneously to these receptors, further evidence of the proximity of MORs and DORs [163]. Thus, both opioid receptors could physically interact to form heterodimers, thereby producing novel signalling elements whose ligand binding, pharmacology, and functional properties could differ from that of the individual monomer. Indeed, heterodimerization can promote changes in the selectivity of some GPCRs towards the different G-protein subfamilies and in particular, co-expression of MORs and DORs reduces their coupling to Gi [164,165]. Although individually MORs and DORs are coupled to pertussis toxin-sensitive Gi/o proteins, when these receptors are



**Fig. (3). The removal of G $\beta\gamma$  dimers at PhLPI and 14-3-3 proteins facilitates the stable transfer of opioid-activated G $\alpha$  subunits to certain RGS proteins.** The reduction of G $\beta\gamma$  dimers brought about by the action of CaMKII on PhLPI increases the number of separated G $\alpha$ GDP subunits. Given that G $\beta\gamma$  dimers are essential to rescue the G $\alpha$ GDP subunits from their GAPs the RGS proteins, their sequestering increases the presence of G $\alpha$  subunits at RGS proteins where the action of kinases like PKC stabilize their binding for long periods of time. The removal of G $\beta\gamma$  dimers at PhLPI facilitates the retention of G $\alpha$  subunits at RGS proteins, and both processes act together to promote the desensitization of MORs.

co-expressed signalling can be observed in the presence of pertussis toxin, suggesting that MOR-DOR heteromers can now extend their regulation to pertussis toxin-insensitive Gz and Gq/11 proteins [165-167].

Thus, although morphine preferentially activates the MOR, the DOR and its endogenous ligands appear to be intimately involved in the chronic and acute responses to morphine. A number of pharmacological studies have demonstrated that MORs and DORs interact and influence each other's behaviour [168]. Notably, DOR antagonists diminish the development of morphine tolerance and dependence [169, 170], and the selective reduction of DORs also attenuates the development of morphine tolerance and dependence [171, 172]. Reduced analgesic tolerance to morphine was also observed using mixed mu-agonist/delta-antagonist such DIPP-NH2[ $\psi$ ] [173] or SoRI9409 [174]. Notably, chronic morphine administration increases the cell surface expression of DORs [175]. Whereas the mechanism behind this phenomenon is still unclear, this increase in DOR trafficking would modify the MOR/DOR ratio at the cell surface, thereby increasing the development of morphine tolerance [176].

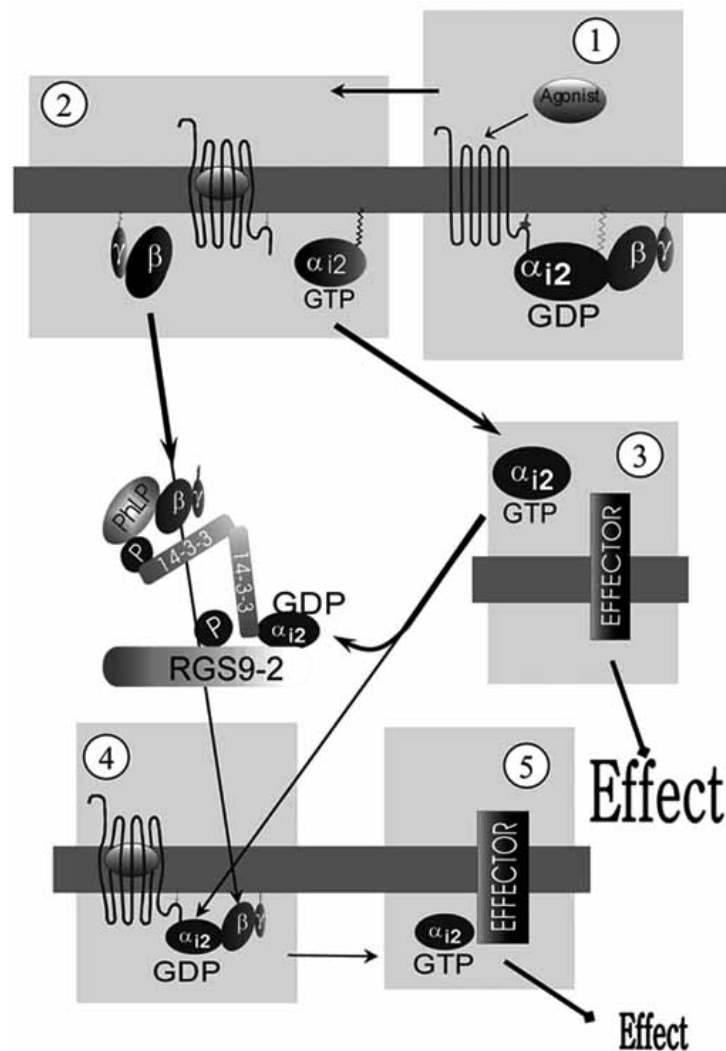
### 5. DOES THE PROPOSED MODEL EXPLAIN THE REDUCED MORPHINE TOLERANCE OBSERVED WHEN CERTAIN SIGNALLING PATHWAYS ARE BLOCKED?

The use of MOR, DOR and NMDAR agonists and antagonists, of kinase/phosphatase activators and inhibitors, in conjunction with the knock-out and knock-down of these and other signalling proteins has provided valuable information on the mechanisms implicated in the development of MOR tolerance to agonists in nervous tissue. One must certainly be cautious as to whether adaptation following some of these

molecular modifications might induce the triggering of signalling pathways other than those normally implicated in the manifestation of opioid tolerance. Notwithstanding, we feel confident that the signalling proteins considered here are relevant because they have been identified by different researchers and generally, using different experimental approaches.

#### 5.1. Inhibition of PKC (PKA)

Impairing PKC activity affects various critical points in this regulatory loop, impeding both the potentiation of NMDAR currents through the activation of Src, and the phosphorylation of NR1 subunits (Fig. 1). Under these conditions the Ca<sup>2+</sup>-CaM complex now binds to the C1 region of NR1 C terminal domain, reducing the open probability of the channel and decreasing the entrance of Ca<sup>2+</sup>. The Ca<sup>2+</sup>-CaM complex activates the PMCA2a isoform of the Ca<sup>2+</sup> pump and the cytosolic Ca<sup>2+</sup> content is rapidly reduced. In the absence of PKC activity, the levels of cytosolic Ca<sup>2+</sup> are low, like those of the Ca<sup>2+</sup>-CaM complexes, with free CaM re-associating with calpactins. Moreover, inhibited PKC will fail to phosphorylate the kinase-enhanced protein phosphatase type 1 inhibitor (KEPI), which is upregulated by acute and chronic morphine [177]. This PKC-activated KEPI and the PKA-activated Inhibitor 1 of PP1 will increase the auto-phosphorylation of CaMKII at Thr286 and thus, maintain this serine/threonine kinase active (see 3.2). Thus, the activity of CaMKII is low when PKC and/or PKA are inhibited, and no sequestering of G proteins is consolidated at PhLPI and RGS proteins (Figs. 2,3). Together, these events probably disrupt the upregulation of NMDAR activity induced by agonist-activated MORs. Accordingly, no tolerance to morphine develops [4, 8, 71, 72, 76, 78, 85].



**Fig. (4).** Schematic flowchart of MOR desensitization caused by the reduction of the regulated G proteins. (1). Activation of MORs segregates the G proteins into GαGTP and Gβγ dimers (2). These signalling proteins regulate their corresponding effectors producing a measurable effect (3). A portion of the Gβγ dimers are sequestered by PhLP1 and 14-3-3 proteins and then the number of Gβγ dimers available to reform the trimeric Gαβγ proteins diminishes (4). Similarly, the GαGTP subunits are deactivated to GαGDP by the action of their GAPs the RGS proteins. The reduction of free Gβγ dimers brought about by PhLP1 facilitates the stable binding of a fraction of the GαGDP subunits at the RGS-R7 proteins. This coordinated sequestering of G protein subunits causes a reduction in the G proteins available to be regulated by MORs (4) and as such, the effects of the agonist are of a smaller magnitude (5).

## 5.2. Antagonism of NOS

The Ca<sup>2+</sup>-CaM complexes generated by the coordinated activity of NTRKs and PKCs on NMDARs also activate neural NOS, which in turn increase the activity of NMDAR and other glutamate receptors. The inhibition of NOS reduces the activity of NMDAR and consequently the activation of CaMKII (Fig. 2). In these circumstances, the MORs maintain their control on the G proteins and no tolerance to morphine analgesia develops [26, 27, 178].

## 5.3. Antagonism of NMDARs

In the presence of the NMDA antagonist, MK801, the signals originated at GPCRs could still activate Src and as a result, some degree of Tyr phosphorylation of NR2 subunits is observed [57]. Similarly, GPCR-activated PKCs are also able to Ser phosphorylate NR1 subunits [179]. However, there is no activation of NMDAR currents and in the absence

of CaMKII activation, the MORs still regulate G proteins (Figs. 2,3). Accordingly, little tolerance to morphine is observed [25, 32-34, 180]. A similar outcome occurs after experimental knockdown of the NMDAR NR1 subunit [181].

## 5.4. Inhibition of CaMKII

This inhibition directly blocks the step that leads to the sequestering of MOR-activated signal transduction elements by PhLP1 and RGS proteins (Figs. 2,3). Thus, the MORs still regulate G proteins and morphine tolerance does not develop [99, 100, 155, 182, 183]. In these circumstances the signals originated at the activated MORs succeed in activating the NMDAR, NTRK-mediated Tyr phosphorylation of NR2 subunits, and PKC-mediated Ser phosphorylation of NR1 (as well as that of calpactins). Indeed, during the time-course of morphine-produced analgesia, KN93, an inhibitor of CaMKII, produces only a temporal inhibition of this kinase [105].

### 5.5. Downregulation of RGS Proteins

The experimental knockdown of members of the RGS-R7 subfamily disrupts the sequestering of MOR-activated  $G\alpha$  subunits (Fig. 3). Because  $G\alpha$ GDP subunits and PhLPI compete for  $G\beta\gamma$  dimers, the increased presence of  $G\alpha$ GDP subunits in the MOR environment prevents PhLPI from sequestering  $G\beta\gamma$  dimers at 14-3-3 proteins. The MOR maintains its control on G proteins and morphine tolerance does not develop [7,17,19-21,117-119,184].

The regulation exerted by RGS-R7 proteins on MOR function is clearly distinct from that of RGS14. Whereas, members of the RGS-R7 family participate in the desensitization of MORs through the sequestering of morphine-activated  $G\alpha$  subunits, RGS14 restrains the access of GRKs to phosphorylate specific residues in the cytosolic C terminus of MORs. Upon removal of RGS14, the morphine-activated MORs are phosphorylated by GRKs and endocytosed. Their recycling back to the membrane resensitises the response to morphine and thus, tolerance develops much slowly [121]. However, the impairment of RGS-R7 function does not promote the phosphorylation and internalization of the MORs but rather prevents the sequestering of the  $G\alpha$  subunits. Accordingly, MORs maintain their regulation of G proteins and no tolerance to morphine develops [21].

### 5.6. Antagonism of DOR Function

The consistent observation that antagonism of DORs reduces morphine tolerance [163, 169-174] indicates that this receptor negatively regulates MOR function. Considering that these opioid receptors form dimers in the spinal cord and PAG [110, 162], and that the activation of MORs increases the expression of surface DORs [175, 176], this regulation could arise through the formation of MOR/DOR heterodimers. The finding that DORs can associate with RGS9 proteins [112] suggests that agonists of this opioid receptor could recruit these RGS proteins to sequester MOR-activated  $G\alpha$ GTP subunits. The diminished opioid tolerance found in preproenkephalin knock-out mice supports this idea [185]. A similar model has also been proposed for RGS-R7 proteins in the cross-regulation of other GPCRs [186, 187]. Nevertheless, further work is required to substantiate this possibility.

### 6. INTERNALIZATION VS NO INTERNALIZATION OF MORs

Whilst the efficient internalization of MORs diminishes the effects of opioid agonists such as DAMGO, neural cells appear to have developed specific mechanisms to control GPCR function when agonists are poor inducers of receptor internalization. In fact, morphine promotes little internalization of MORs but desensitizes a large fraction of these receptors that remain in the cell membrane. Morphine-induced MOR desensitization is clearly connected with NMDA glutamate receptor activity, the subsequent activation of CaMKII, and with the sequestering of the MOR-regulated components of G-proteins by PhLPI and RGS proteins. Thus, PKC has been implicated in the MOR desensitization to morphine, but less so in the effects of DAMGO [188]. Indeed, antagonists of NMDA receptors impede the development of tolerance to morphine antinociception but have little

effect on that promoted by DAMGO [35]. Whereas morphine causes a gradual and long-lasting activation of CaMKII, DAMGO brings about an intense but brief activation of this kinase [46, 121, 189] and it does not consolidate the transfer of  $G\alpha$  subunits to RGS proteins [11]. These differences indicate that DAMGO and related opioids initiate signals that trigger the activation of NMDA-CaMKII pathway. However, these signals are disrupted by MOR internalization and the sequestering of the transduction regulated by these receptors does not occur [11, 121]. DAMGO produces a low level of tolerance given that the MORs belong to the class of GPCRs that are rapidly dephosphorylated and recycled after internalization [9, 10]. Nevertheless, a fraction of these internalized receptors are sorted to lysosomes and undergo proteolytic degradation [190]. It must be borne in mind when using demanding protocols that these agonists deplete the surface MORs before de novo synthesis can restore the system, which also leads to inescapable desensitization [11,35, 191, 192].

Recent studies have shown that tolerance to morphine in mature neurons develops through a two step process [11]. Firstly, the MORs become depleted of  $G\alpha$  subunits and they develop strong antinociceptive tolerance. Subsequently, additional doses of this agonist provoke the phosphorylation and recycling of the MORs, with the consequence that the reduced effects that remain after the first dose now desensitize more slowly.

### 7. CONCLUDING REMARKS

In the nervous system, the differences between opioid agonists in producing MOR endocytosis and tolerance may be related to their capacity to activate NMDAR currents. Most of the pharmacological approaches that reduce morphine tolerance act on different links in the same chain of events that controls MOR function. The final step of these related processes is the sequestering of the G proteins that are regulated by the MORs. So far the pieces of this puzzle that offer better possibilities for specific intervention are the surface MORs, DORs and NMDARs. The other signalling proteins implicated in the MOR and NMDAR regulatory loop also participate in a variety of regulatory processes. Thus, their selectivity in any given physiological process derives from the moment they are activated/inactivated, the time they begin to regulate another signalling protein, and their translocation to an adequate environment. Notwithstanding, by influencing parts of the regulatory mechanisms that desensitize the MORs in mature neurons, this knowledge may enable us to develop therapeutic approaches that prevent morphine tolerance or that rescue the system. Recent research directed towards understanding the cross regulation between MORs and DORs, and the negative influence of DORs on MOR function could provide clues that will help to develop ligands that take advantage of the selectivity provided by the binding sites of these receptors. Similarly, this knowledge may help in the search for selective activators and antagonists of NMDARs, affecting different NR2/3 subunits [193]. These substances will be useful to unveil which NMDARs classes are relevant in producing MOR tolerance, and selectivity we be gained by designing therapeutic approaches in which these NMDARs are acted upon.

**Key Learning Objectives:**

In recent years, a series of pharmacological approaches have consistently been described as effectively attenuating or reversing morphine antinociceptive tolerance. On the basis of this ample information, the current review intends to bring together and ensemble the most important molecular elements of the regulatory machinery on which these drugs may act to diminish morphine-induced desensitization of the MORs.

**Future Research Directions:**

Most of the pharmacological approaches that reduce morphine tolerance appear to act on different links in the same chain of events that controls MOR function. Hence, it would be both of value and interest: to identify additional links in this chain in order to improve our understanding of the processes underlying the development of morphine tolerance in the nervous system. In particular, which classes of NMDARs are relevant to produce and/or maintain MOR tolerance; to analyze the capacity of therapeutically relevant opioids to promote MOR endocytosis and to activate the regulatory events that lead to MOR desensitization. This information would identify appropriate pharmacological targets to attempt to diminish tolerance.

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